HUMAN VACCINES & IMMUNOTHERAPEUTICS
2019, VOL. 15, NO. 10, 2359–2377
https://doi.org/10.1080/21645515.2019.1651140

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REVIEW

Vaccines against Ebola virus and Marburg virus: recent advances and promising candidates

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ABSTRACT

The filoviruses Ebola virus and Marburg virus are among the most dangerous pathogens in the world. Both viruses cause viral hemorrhagic fever, with case fatality rates of up to 90%. Historically, filovirus outbreaks had been relatively small, with only a few hundred cases reported. However, the recent West African Ebola virus outbreak underscored the threat that filoviruses pose. The three year-long outbreak resulted in 28,646 Ebola virus infections and 11,323 deaths. The lack of Food and Drug Administration (FDA) licensed vaccines and antiviral drugs hindered early efforts to contain the outbreak. In response, the global scientific community has spurred the advanced development of many filovirus vaccine candidates. Novel vaccine platforms, such as viral vectors and DNA vaccines, have emerged, leading to the investigation of candidate vaccines that have demonstrated protective efficacy in small animal and nonhuman primate studies. Here, we will discuss several of these vaccine platforms with a particular focus on approaches that have advanced into clinical development.

ARTICLE HISTORY
Received 25 April 2019
Revised 14 July 2019
Accepted 27 July 2019

KEYWORDS
Ebola virus; Marburg virus; filovirus; vaccines

Introduction

The *Filoviridae* family of viruses is composed of enveloped RNA viruses with nonsegmented, negative-sense genomes. Filoviruses are divided into three serologically distinct genera: *Ebolavirus, Marburgvirus*, and *Cuevavirus*.[1] The *Ebolavirus* genus is composed of six species including *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Tai Forest Virus* (TAFV), *Reston ebolavirus* (RESTV), *Bundibugyo ebolavirus* (BDBV), and *Bombali ebolavirus* (BOMV). Disease cases have been reported for EBOV, SUDV, TAFV, and BDBV, but RESTV does not appear to cause human disease. BOMV has not been reported to cause disease, but data is incomplete. EBOV is considered the most lethal species in the genus, with a lethality range of 60–90% in human outbreaks.[2] The *Marburgvirus* genus currently includes a single viral species, *Marburg marburgvirus*, which is lethal in 70–85% of cases. It is defined by two viruses, Marburg virus (MARV) and Ravn virus (RAVV). *Cuevavirus* genus has a single viral species, *Lloviu cuevavirus*, and one defined virus, Lloviu virus (LLOV). Filovirus infection frequently presents as severe hemorrhagic fever in humans, with symptoms including fever, anorexia, diarrhea, hemorrhaging, and petechial rash although the World Health Organization (WHO) recently reported that several Ebola virus disease cases did not result in hemorrhaging.[1,3] The high mortality rate and possibility for person to person transmission of EBOV and MARV have led to their inclusion in the WHO's Blueprint list of priority diseases.

The potential for EBOV to cause a large-scale outbreak became abundantly clear during the 2013 West African Outbreak. Beginning in December 2013 with the infection of a 2 year old child in Guinea, the disease spread to neighboring Liberia and Sierra Leone.[4] Smaller outbreaks were reported in Mali and Nigeria, and individual travel related cases occurred in the USA, Italy, Spain, and the United Kingdom (UK), triggering worldwide alarm.[5] In total, 28,646 EBOV cases and 11,323 deaths were reported, making this the largest and deadliest EBOV outbreak in history.[6] The outbreak was finally declared over on 9 June, 2016. Several factors helped undermine efforts to control the outbreak, including the lack of specific vaccines, drugs, or therapeutic treatments for EBOV infection. A concerted effort was quickly made to develop an effective filovirus vaccine and multiple clinical trials were launched to cope with the epidemic.

Previously, several filovirus vaccines were tested in small rodent models, but few candidates had moved into advanced development. Lack of funding, a limited commercial market, and a poor understanding of the correlates of protective immunity hindered filovirus vaccine design efforts. Immune profiles collected from survivors of various filovirus outbreaks suggest the humoral response at least partially correlates with protection. Early development of anti-EBOV IgM antibodies, followed by the development of anti-EBOV IgG, strongly correlates with survival. Conversely, nonsurvivors frequently do not develop high-level IgG responses.[7] The presence of neutralizing antibodies following EBOV, SUDV, BDBV, and MARV infection,[8-12] and the ability to treat nonhuman primates (NHPs) with monoclonal antibodies following filovirus

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challenge,[13,14] suggest that neutralizing antibodies contribute to protection. Recent reports have also shown that potent T cell responses correlate with survival. McElroy et al. demonstrated that EBOV survivors exhibited persistently high levels of IFN-γ^+ and TNF-α^+ $CD4^+$ and $CD8^+$ T cells following exposure.[15] A similar study conducted by Stonier et al. reported that MARV survivors developed multivariate $CD4^+$ T cell populations that had a T_h1 skew, but that $CD8^+$ T cell levels were low and exhibited little effector function.[16] Additionally, vaccine protective efficacy studies appear to show little cross-protection between viral species as detailed below. Taken together, these data suggest that each filovirus species may present a unique challenge to vaccine design, and that a multi-species vaccine approach is necessary to protect against each antigenically distinct species of the filovirus family.

As NHPs are considered the "benchmark" model for both EBOV and MARV, the protective efficacy of many new vaccine candidates remains unexplored. Filovirus infected rhesus and cynomolgus macaques present with similar disease symptoms as humans, making them an invaluable resource for vaccine studies. In this review, we will describe early proof of concept rodent data to illustrate the developmental approach for some vaccine platforms. However, the main focus will be on NHP studies (Tables 1 and 2) and clinical trials (Tables 3 and 4) where data are available. Unless specifically noted, animals were challenged with the homologous filovirus species.

Inactivated virus and subunit protein vaccines

As with most infectious diseases, the first vaccine platform explored for filoviruses was an inactivated whole-virus vaccine directed against EBOV. Results from these early studies were inconsistent. Initially, two separate inactivated whole-virus vaccines were generated using either heat or formalin treatment of EBOV virions.[50] Both vaccines were tested in the guinea pig EBOV challenge model. While both inactivation methods yielded protective vaccines, several of the challenged guinea pigs developed symptoms of febrile disease following EBOV challenge, including fever and weight loss. Further development has led to more novel methods for generating inactivated filovirus vaccines. Warfield et al. inactivated EBOV virions by treatment with the photoinducible alkylating agent 1, 5 iodonaphthylazide (INA).[51] INA inactivation produced virions that were morphologically

Table 1. Protective efficacy studies of EBOV vaccines in NHPs.

Vaccine Modality	Challenge Virus	Vaccine Doses	Time to Challenge (d)	Survival (%)	Ref. #
Inactivated Virus					
Inactivated EBOV	EBOV	3	43	25	17
Inactivated EBOV	EBOV	2	56	0	18
EBOVΔVP30	EBOV	1, 2	28	100	19
Replication Incompetent Vaccines					
Virus-like Replicon Particle (VRP)					
VRP-EBOV GP, VRP-EBOV NP, VRP-EBOV GP + NP	EBOV	3	49	0	17
VRP-EBOV, VRP-SUDV	EBOV	1	28	100	20
VRP-EBOV, VRP-SUDV	SUDV	1	28	100	20
Adenovirus Vector					
Ad5.EBOV GP + Ad5. EBOV NP	EBOV	2	7	100	21
Ad5.EBOV GP + Ad5. EBOV NP, Ad5.EBOV GPΔTM + Ad5.EBOV NP	EBOV	1	28	100	22
Ad5.EBOV GP	EBOV	1	28	100	23
Ad35.EBOV GP	EBOV	1	28	11	24
Ad26.EBOV GP	EBOV	1	28	75	24
Ad26.EBOV GP + Ad35. EBOV GP	EBOV	2	28	100	24
Ad26.EBOV GP + Ad35. EBOV GP	EBOV	2	56	75	25
ChAd3.EBOV GP	EBOV	1	330	50	26
ChAd3.EBOV GP	EBOV	2	56	33	26
CAdVax-Panfilo	EBOV	2	42	100	27
CAdVax-Panfilo	EBOV	2	41	100	28
CAdVax-Panfilo	SUDV	2	41	100	28
Vaccinia Virus Vectors					
VACV-GP	EBOV	3	45	0	17
MVA-EBOV GP/VP40	EBOV	1	56	100	29
MVA-EBOV GP/VP40	EBOV	2	28	100	29
DNA					
EBOV GP	EBOV	3	56	83	30
EBOV GP + SUDV GP + MARV GP + RAVV GP	EBOV	3	56	20	30
EBOV GP	EBOV	2, 3	28	50, 100	31
VLP					
EBOV GP/NP/VP40 + RIBI	EBOV	3	28	100	32
EBOV GP/NP/VP40 + QS-21	EBOV	2	28	100	33
EBOV GP/VP40 + QS-21	EBOV	2	28	40	33
Replication Competent Vaccines					
Recombinant Cytomegalovirus					
RhCMV-EBOV GP	EBOV	2	28	75	34
Human Parainfluenza Virus					
HPIV3-EBOV GP + NP	EBOV	1, 2	28	88	35
HPIV3-EBOV GP + NP	EBOV	1, 2	27	100	36
Recombinant Rabies Virus					
BNSP333-coEBOV GP	EBOV	1	70	100	37
Inactivated BNSP333-coEBOV GP	EBOV	2	42	50	37
BNSP333-coEBOV GP	EBOV	2	28	100	38
Recombinant Vesicular Stomatitis Virus					
VSV-EBOV	EBOV	1	28	100	39
VSV-EBOV + VSV-SUDV + VSV-MARV	EBOV	1	28	75	40
VSV-EBOV + VSV-SUDV + VSV-MARV	SUDV	1	28	50	40
VSV-EBOV + VSV-SUDV + VSV-MARV	TAFV	1	28	100	40
VSV-EBOV	BDBV	1	28	75	41
VSV-TAFV	BDBV	1	28	30	41
Mixed Modality					
ChAd3.EBOV GP + MVA-BN-Filo	EBOV	2	28	100	26
Ad26.EBOV GP + MVA-BN -Filo	EBOV	2	28	100	25
EBOV GP DNA + Ad5. EBOV GP	EBOV	4	56	100	42
EBOV GP DNA + Ad5. EBOV GP	EBOV	5	7	100	43

Table 2. Protective efficacy studies of MARV vaccines in NHPs.

Vaccine Modality	Challenge Virus	Vaccine Doses	Time to Challenge (d)	Survival (%)	Ref. #
Inactivated Virus					
Inactivated MARV	MARV	2	21	50	44
Replication Incompetent Vaccines					
Virus-like Replicon Particle (VRP)					
VRP-MARV GP	MARV	3	35	100	45
VRP-MARV NP	MARV	3	35	67	45
VRP-MARV GP + VRP-MARV NP	MARV	3	35	100	45
Adenovirus Vector					
Ad5.MARV GP	MARV	1	28	100	46
CAdVax-panFilo	MARV	2	42	100	27
DNA					
MARV GP	MARV	4	21	100	46
MARV GP	MARV	3	28	67	47
MARV GP	MARV	3	56	83	30
EBOV GP + SUDV GP + MARV GP + RAVV GP	MARV	3	56	100	30
VLP					
MARV GP, MARV GP + NP	MARV	3	28	100	48
MARV GP/NP/VP40 + Poly-IC	MARV	3	28	100	49
MARV GP/NP/VP40 + QS-21	MARV	3	28	100	49
Replication Competent Vaccines					
Recombinant Vesicular Stomatitis Virus					
VSV-MARV	MARV	1	28	100	39
VSV-EBOV + VSV-SUDV + VSV-MARV	MARV	1	28	75	40
Mixed Modality					
MARV GP DNA + Ad5.MARV GP	MARV	4	42	100	46

Table 3. Clinical trials with patients receiving a single filovirus vaccine platform candidate.

NCT Number	Viral Target	Vaccine	Phase	Study Dates	Country
NCT00374309	EBOV	Ad5.EBOV	1	Study Start: Sept. 2006 Study Completion: May 2009	USA
NCT02231866	EBOV SUDV	ChAd3.EBOV+ChAd3.SUDV ChAd3.EBOV	1	Study Start: August 2014 Study Completion: April 2017	USA
NCT02485301	EBOV	ChAd3.EBOV Placebo	2	Study Start: July 2015 Study Completion: Dec. 2016	Cameroon Mali Nigeria Senegal
NCT02548078	EBOV	ChAd3.EBOV	2	Study Start: Nov. 2015 Study Completion: May 2017	Mali Senegal
NCT02401373	EBOV	Ad5.EBOV	1	Study Start: March 2015 Study Completion: July 2015	China
NCT02485912	EBOV	ChAd3.EBOV+MVA-EBOV	1	Study Start: July 2015 Study Completion: Jan. 2016	Senegal
NCT02354404	EBOV SUDV	ChAd3.EBOV ChAd3.EBOV+ChAd3.SUDV MVA-EBOV	1	Study Start: Jan. 2015 Study Completion: April 2017	Uganda
NCT02344407	EBOV	VSV-EBOV ChAd3.EBOV Placebo	2	Study Start: Jan. 2015 Study Completion: June 2020	Liberia
NCT02289027	EBOV	ChAd3.EBOV	1/2	Study Start: October 2014 Study Completion: June 2015	Switzerland
NCT02533791	EBOV	Ad5.EBOV Low Dose Ad5.EBOV High Dose	1	Study Start: July 2015 Study Completion: Oct. 2015	China
NCT02344407	EBOV	VSV-EBOV ChAd3.EBOV Placebo	2	Study Start: Jan. 2015 Study Completion: June 2020	Liberia
NCT02575456	EBOV	Ad5.EBOV Placebo	2	Study Start: Oct. 2015 Study Completion: July 2016	Sierra Leone
NCT02326194	EBOV	Ad5.EBOV Low Dose Ad5.EBOV High Dose	1	Study Start: Dec. 2014 Study Completion: July 2015	China
NCT03475056	MARV	ChAd3.MARV	1	Study Start: Oct. 2018 Study Completion: Dec. 2019	USA
NCT00072605	EBOV	EBOV GP DNA	1	Study Start: Nov. 2003 Study Completion: August 2007	USA
NCT00605514	EBOV MARV	EBOV GP DNA MARV GP DNA	1	Study Start: Jan. 2008 Study Completion: June 2010	USA
NCT00997607	EBOV MARV	EBOV GP DNA MARV GP DNA	1	Study Start: Feb. 2010 Study Completion: April 2012	Uganda
NCT02464670	EBOV	EBOV GP DNA	1	Study Start: May 2015 Study Completion: May 2018	USA
NCT02370589	EBOV	EBOV GP VLP EBOV GP VLP+Matrix M	1	Study Start: Feb. 2015 Study Completion: April 2016	Australia

(*Continued*)

Table 3. (Continued).

NCT Number	Viral Target	Vaccine	Phase	Study Dates	Country
NCT02564575	EBOV	HPIV3/EBOV	1	Study Start: August 2015 Study Completion: Nov. 2016	USA
NCT03462004	EBOV	HPIV3/ΔF-HN/EBOVGP	1	Study Start: March 2018 Study Completion: Dec. 2019	USA
NCT02374385	EBOV	VSV-EBOV Placebo	1	Study Start: Nov. 2014 Study Completion: June 2015	Canada
NCT02287480	EBOV	VSV-EBOV	1/2	Study Start: Nov. 2014 Study Completion: Jan. 2016	Switzerland
NCT02296983	EBOV	VSV-ZEBOV	1	Study Start: Dec. 2014 Study Completion: Sept. 2016	Kenya
NCT03161366	EBOV	VSV-EBOV	3	Study Start: May 2018 Study Completion: Nov. 2018	DRC Uganda
NCT02283099	EBOV	VSV-EBOV	1	Study Start: Nov. 2014 Study Completion: Nov. 2015	Germany
NCT02269423	EBOV	VSV-EBOV Placebo	1	Study Start: Oct. 2014 Study Completion: Aug. 2015	Randomized
NCT02280408	EBOV	VSV-EBOV Placebo	1	Study Start: Oct. 2014 Study Completion: Dec. 2015	Randomized
NCT02314923	EBOV	VSV-EBOV Placebo	1	Study Start: Dec. 2014 Study Completion: June 2016	Randomized
NCT02718469	EBOV	VSV-EBOV	1	Study Start: Dec. 2015 Study Completion: Sept. 2016	USA
NCT02378753	EBOV	VSV-EBOV	2/3	Study Start: April 2015 Study Completion: Dec. 2016	Sierra Leone
NCT02503202	EBOV	VSV-EBOV	3	Study Start: Aug. 2015 Study Completion: Sept.2017	Randomized
NCT03333538	EBOV	VSV-EBOV	1/2	Study Start: Nov. 2017 Study Completion: Dec. 2018	Russian Federation
NCT03031912	EBOV	VSV-EBOV	2	Study Start: Aug. 2017 Study Completion: Dec. 2019	Canada
NCT02933931	EBOV	VSV-EBOV		Study Start: Nov. 2016 Study Completion: April 2020	Unknown
NCT02788227	EBOV	VSV-EBOV	2	Study Start: Jan. 2016 Study Completion: Oct. 2022	USA Canada

indistinguishable from live virus. The INA EBOV vaccine protected 80% of vaccinated mice from lethal EBOV challenge, likely through the elicitation of EBOV-specific antibodies and $CD8^+$ T cells. Similar levels of protection were measured in inactivated MARV studies for the Musoke and RAVV strains. Irradiated, whole MARV virions completely protected guinea pigs from homologous challenge.[52,53] Despite these successes, the protective efficacy seen in small animal models was not sustained in NHPs. Vaccination with gamma irradiated EBOV particles caused all NHPs to seroconvert and develop marginal levels of neutralizing antibodies. However, these vaccines failed to protect NHPs from EBOV challenge, even when encapsulated within liposome vesicles to improve cellular uptake.[17,18] Likewise, an inactivated MARV vaccine only conferred 50% protection.[44] The limited efficacy demonstrated in these reports has prevented further inactivated vaccine studies from being conducted in NHPs.

An attenuated virus vaccine approach was tested using a replication-defective EBOV lacking the VP30 transcription factor (EBOVΔVP30), rendering it unable to propagate within the host.[54,55] Although EBOVΔVP30 was completely protective in mice and guinea pigs,[55] safety concerns remained due to the possibility of reversion leading to viral replication. These concerns led Marzi et al. to inactivate the EBOVΔVP30 vaccine by treatment with hydrogen peroxide (H_2O_2). Mirroring small animal model results, a prime/boost vaccination strategy for both the EBOVΔVP30 and the H_2O_2-EBOVΔVP30 vaccines yielded robust IgG responses and completely protected NHPs from EBOV challenge.[19] Similarly, a single dose of the EBOVΔVP30 vaccine protected NHPs from challenge, but clinical signs of illness were observed suggesting that a prime/boost approach is necessary. The requirement for BSL-3 production has hindered H_2O_2-EBOVΔVP30's utility though.[56]

As with the inactivated whole-virus vaccines, limited data detailing the protective efficacy of subunit vaccines have been published. Hevey et al. showed that a recombinant baculovirus produced MARV glycoprotein (GP) vaccine lacking the transmembrane domain protected 4/5 guinea pigs from homologous Musoke challenge.[52] However, no guinea pigs survived challenge with the heterologous RAVV species, suggesting that subunit baculovirus-derived vaccines have limited cross-protective efficacy. Other studies involving an adjuvanted trivalent EBOV vaccine composed of recombinant GP, VP24, and VP40 proved that complete protection can be achieved in mice.[57] Microneedle patch intradermal delivery of EBOV GP adjuvanted with saponins protected mice from EBOV challenge.[58,59] Additionally, recent advancements allowing for the fusion of EBOV extracellular domains to the Fc fragment of human immunoglobulin have proven somewhat more immunogenic. When delivered in combination with poly-ICLC adjuvant, this vaccine completely protected guinea pigs from EBOV challenge.[60] These small animal studies suggest that recombinant subunit vaccines may be a possible route for eliciting protective immunity against filovirus infection, but there

Table 4. Clinical trials with patients receiving combined filovirus vaccine candidates.

NCT Number	Viral Target	Vaccine	Phase	Study Dates	Country
NCT03140774	EBOV SUDV TAFV MARV	Ad26.EBOV+MAV-BN-Filo VSV-EBOV	1/2	Study Start: May 2017 Study Completion: July 2020	UK
NCT02313077	EBOV SUDV TAFV MARV	MVA-BN-Filo Ad26.EBOV Placebo	1	Study Start: Dec. 2014 Study Completion: March 2016	UK
NCT02267109	EBOV SUDV TAFV MARV	ChAd3.EBOV+MVA-BN-Filo ChAd3.EBOV+Placebo	1	Study Start: Oct. 2014 Study Completion: April 2016	Mali
NCT02376426	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo	1	Study Start: March 2015 Study Completion: June 2016	Kenya
NCT02543268	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo	3	Study Start: Sept. 2015 Study Completion: July 2016	USA
NCT02368119	EBOV SUDV TAFV MARV	ChAd3.EBOV+MVA-BN-Filo	1	Study Start: March 2015 Study Completion: Sept. 2016	Mali
NCT02376400	EBOV SUDV TAFV MARV	MVA-BN-Filo Ad26.EBOV Placebo	1	Study Start: April 2015 Study Completion: Sept. 2016	Tanzania Uganda
NCT02543567	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo	3	Study Start: Sept. 2015 Study Completion: Nov. 2016	USA
NCT02408913	EBOV SUDV TAFV MARV	MVA-BN-Filo ChAd3.EBOV+MVA-BN-Filo	1	Study Start: March 2015 Study Completion: April 2017	USA
NCT02325050	EBOV SUDV TAFV MARV	MVA-BN-Filo Ad26.EBOV Placebo	1	Study Start: Jan. 2015 Study Completion: May 2017	USA
NCT02451891	EBOV	MVA-EBOV ChAd3.EBOV	1	Study Start: April 2015 Study Completion: Aug. 2017	UK
NCT02240875	EBOV SUDV TAFV MARV	ChAd3.EBOV+MVA-BN-Filo	1	Study Start: Sept. 2014 Study Completion: Aug. 2017	UK
NCT02495246	EBOV	ChAd3.EBOV Ad26.EBOV	1	Study Start: Sept. 21, 2015 Study Completion: Aug. 2017	UK
NCT02416453	EBOV SUDV TAFV MARV	MVA-BN-Filo Ad26.EBOV Placebo	2	Study Start: June 2015 Study Completion: Jan. 2018	France UK
NCT02598388	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo	2	Study Start: Jan. 2016 Study Completion: Dec. 2018	USA Kenya Mozambique Nigeria Tanzania Uganda
NCT02354404	EBOV SUDV	ChAd3.EBOV ChAd3.EBOV+ChAd3.SUDV MVA-EBOV	1	Study Start: Jan. 2015 Study Completion: April 2017	Uganda
NCT02891980	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo	1	Study Start: March 2017 Study Completion: March 2019	USA
NCT02876328	EBOV SUDV TAFV MARV	Ad26.EBOV MVA-BN-Filo VSV-EBOV Placebo	2	Study Start: March 2017 Study Completion: March 2019	Guinea Liberia Mali Sierra Leone
NCT02564523	EBOV SUDV TAFV MARV	Ad26.EBOV+MVA-BN-Filo Placebo	2	Study Start: Nov. 2015 Study Completion: March 2019	Burkina Faso Côte D'Ivoire Kenya Uganda

(*Continued*)

Table 4. (Continued).

NCT Number	Viral Target	Vaccine	Phase	Study Dates	Country
NCT02509494	EBOV SUDV TAFV MARV	ChAd3.EBOV+MVA-BN-Filo	3	Study Start: Sept. 2015 Study Completion: Aug. 2019	Sierra Leone
NCT02911415	EBOV	VSV-EBOV+Ad5.EBOV	1	Study Start: Sept. 2016 Study Completion: Dec. 2017	Russian Fed.
NCT03072030	EBOV	VSV-EBOV+Ad5.EBOV Placebo	4	Study Start: Aug. 2017 Study Completion: Dec. 2019	Guinea Russian Fed.
NCT02344407	EBOV	VSV-EBOV ChAd3.EBOV Placebo	2	Study Start: Jan. 2015 Study Completion: June 2020	Liberia
NCT03140774	EBOV SUDV TAFV MARV	Ad26.EBOV+MAV-BN-Filo VSV-EBOV		Study Start: May 2017 Study Completion: July 2020	UK
NCT03583606	EBOV SUDV TAFV MARV	ChAd3.EBOV+MVA-BN-Filo	1	Study Start: Oct. 2018 Study Completion: Aug. 2020	USA
NCT02661464	EBOV SUDV TAFV MARV	Ad26.ZEBOV MVA-BN-Filo	3	Study Start: May 2016 Study Completion: April 2023	USA Burkina Faso Côte D'Ivoire France
NCT02661464	EBOV SUDV TAFV MARV	Ad26.ZEBOV MVA-BN-Filo	3	Study Start: May 2016 Study Completion: April 2023	USA Burkina Faso Côte D'Ivoire France

remains a need for further testing in a more relevant disease model such as NHPs.

Replication incompetent vaccines

Non-replicating vaccines are generally more immunogenic than the whole-inactivated or subunit vaccines. These vaccines are usually considered safer than replicating vaccines, as they do not carry a risk of reversion to virulence. Here, we will discuss some of the more promising replication incompetent filovirus vaccines. Additional detailed reviews covering non-replicating vaccines have been published, including Hoenen et al. and Mire and Geisbert.[61,62]

Virus–like replicon particles (VRPs)

Virus-like replicon particles (VRPs) are generated by using a Venezuelan equine encephalitis virus (VEEV) vector to produce replication-incompetent particles capable of entering a host cell. VEEV is a nonsegmented, positive-sense RNA virus of the genus *Alphavirus* in the family *Togaviridae*. The genome is divided into two open reading frames encoding either structural proteins or nonstructural proteins. For VRP production, key VEEV structural proteins can be substituted for with a viral antigen of interest (i.e. substitute filovirus gene in place of VEEV gene). This limits the particle to a single round of infection while retaining the ability to produce ample amounts of antigen within the host cell.[63] For both EBOV and MARV, the antigen encoded with the VRP is typically GP due to it being a potent target for protective antibody responses,[64-66] but VRPs expressing EBOV GP, nucleoprotein (NP), VP24, VP30, VP35, and VP40 have been reported.[67]

Early studies conducted at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) illustrated the promise of VRP filovirus vaccines. Pushko et al. showed that VRPs encoding either EBOV GP or NP are immunogenic and protective in mice,[63] but that only the VRP-EBOV GP was protective in guinea pigs[68] and NHPs.[17] Olinger et al. later established that VRP-EBOV can induce protective cytotoxic T cell responses in mice.[67] In 2013, Herbert et al. published a report detailing protection against EBOV and SUDV challenge in NHPs. In this study, two groups of three NHPs were given a single vaccination with a combination of VRPs encoding SUDV GP and EBOV GP.[20] One group was then challenged with SUDV, and the other was challenged with EBOV. All vaccinated NHPs survived challenge, and showed no clinical signs of disease. The animals were then back-challenged with the heterologous virus. The EBOV back-challenged NHPs survived secondary challenge without developing clinical signs of disease. However, one of the SUDV back-challenged animals exhibited clinical disease, to include fever, increases in liver enzymes, and viremia, before succumbing to challenge (67% survival rate). The data published by Herbert and colleagues suggest that a combination VRP-SUDV GP and VRP-EBOV GP vaccine can elicit protection against SUDV and EBOV challenge, but that VRP-SUDV GP is insufficient for *pan-Ebolavirus* immunity.

Similar efficacy has been demonstrated for MARV. Guinea pig studies with VRPs encoding MARV VP40, VP35, and VP24 provided partial protection, as did vaccination with a VRP encoding a transmembrane deleted GP construct.[45] These promising results led to further efficacy studies in NHPs. Cynomolgus macaques were vaccinated three times with VRP-MARV GP, VRP-MARV NP, or a combination of both. Vaccination with VRP-MARV GP alone or in combination with VRP-MARV NP provided complete protection from

homologous MARV challenge with minimal clinical disease symptoms. As with the VRP-EBOV NP vaccine, VRP-MARV NP was much less efficacious, with one NHP succumbing to MARV disease, and two survivors showing severe disease.[45] More recently, Ren et al. constructed an alphavirus replicon using the Semliki forest virus replicon vector, DREP.[69] Vaccination with DREP-GP and DREP-VP40 induced antigen-specific IgG and IFN-γ^+ $CD8^+$ T cells in mice. A bivalent DREP vaccine expressing EBOV GP and SUDV GP elicited similar levels of anti-GP IgG in mice.[70] The protective efficacy of the DREP vectored vaccines remain to be tested.

Adenovirus vectors

An increasingly attractive non-replicating filovirus vaccination strategy is the use of recombinant adenovirus vectors. Replication deficient adenovirus vectors are highly immunogenic, and can generate robust B and T cell responses to viral antigens.[71,72] Adenoviruses have been developed as vaccine vectors for multiple antigens, and considerable research has been conducted to examine the protective efficacy of adenovirus-based filovirus vaccines. Replication defective adenovirus vectored EBOV vaccines lack the E1 and E3 adenovirus gene segments. Instead, EBOV GP has been inserted into the E1 position of the adenovirus genome, resulting in significant levels of EBOV GP production. Initial adenovirus vector designs used human serotypes such as Ad5, causing some concern due to pre-existing AdHu5 immunity in human populations.[73,74] Early studies seemed to indicate this was not a significant problem, as vaccination with the Ad5.EBOV GP vaccine not only elicited neutralizing antibodies and IFN-γ producing $CD8^+$ T cells in mice, but it also protected mice from lethal EBOV challenge.[74] Complete protection was also seen in NHPs vaccinated with the Ad5.EBOV GP vaccine.[75] Moreover, vaccination with an Ad5 vector encoding EBOV GP and NP protected NHPs from challenge over a range of vaccine doses.[21,22,75] These results reflect those reported by Geisbert et al. showing that vaccination with Ad5.$MARV_{Angola}$ GP generated high levels of antigen-specific IgG antibodies and cellular immunity.[46] As with the Ad5.EBOV GP vaccine, these immune responses correlated with complete protection from viral challenge. Vaccinated NHPs did not develop clinical signs of MARV disease, with no marked increase in liver enzymes, lymphopenia, or systemic viremia. The first Phase I clinical trial (NCT00374309) was conducted by Ledgerwood et al. in 2010.[76] This trial was a randomized, double-blinded, placebo-controlled, dose-escalating study utilizing a product composed of Ad5.EBOV GP and Ad5.SUDV GP. Patients received either a low dose (2×10^9) or high dose (2×10^{10}) of virus particles by intramuscular (IM) injection. Vaccinees developed antigen-specific humoral and cellular immune responses that were dose dependent as EBOV GP-specific antibody titers and T cell responses were significantly increased in the high dose group. For both dose groups, the most common adverse reaction was a mild, short-lived headache.

Recently, Wu et al. adapted the Ad5.EBOV vaccine to express the GP from the 2013 West African $EBOV_{Makona}$ outbreak and demonstrated the vaccine's protective efficacy in both guinea pigs and NHPs.[23] Most notably, an Ad5.EBOV vaccine encoding Makona GP was shown to be safe and highly immunogenic in Phase I clinical trials conducted in China (NCT02326194) and Sierra Leone (NCT02401373).[77,78] Pointedly though, higher doses of the Ad5.$EBOV_{Makona}$ GP were required to overcome pre-existing Ad5 immunity in the Chinese population. In addition, the immune responses waned after only 4 weeks.[79] A homologous Ad5.$EBOV_{Makona}$ GP boost at 6 months did improve antibody titers several fold, but these results may call the clinical feasibility of the Ad5 vector into question.

In agreement with human Ad5.$EBOV_{Makona}$ GP data, Kobinger et al. demonstrated that pre-exposure of mice to an Ad5 vector expressing an irrelevant antigen interfered with the ability of an Ad5.EBOV GP vaccine to elicit antigen-specific T cells in mice.[74] This was supported by a proof-of-concept study in NHPs demonstrating that previous Ad5 exposure limits the protective efficacy of the Ad5.EBOV GP vaccine.[24] To circumvent the problem of pre-existing immunity, several groups have selected less common strains of adenovirus. In particular, Ad35 and Ad26, which are genetically distinct from Ad5 and are not impacted by Ad5 pre-existing immunity, have been investigated as attractive vector alternatives. Geisbert et al. showed that vaccination with Ad26.EBOV GP or Ad35.EBOV GP induced neutralizing antibodies after vaccination. However, only Ad26.EBOV GP demonstrated any protective efficacy, with 75% of NHPs surviving EBOV challenge.[24] The Ad35 vector did prove effective when delivered as a heterologous boosting vaccination. Priming with an Ad26 vector encoding EBOV GP and $SUDV_{Gulu}$ GP, followed by an Ad35 EBOV GP and $SUDV_{Gulu}$ GP boost, increased GP-specific antibody titers and $CD8^+$ T cells. This vaccination regimen resulted in complete protection from morbidity and mortality following EBOV challenge, providing a possible route for avoiding host adenovirus immunity.[24]

Similarly, Stanley et al., tested an approach for evading pre-existing adenovirus host immunity by using an EBOV GP vaccine based on chimpanzee Ad3 (ChAd3.EBOV GP). When given as a single shot with an equivalent dose to that used for the Ad5.EBOV GP vaccine, the ChAd3.EBOV GP protected 50% of NHPs from viral challenge.[26] Interestingly, a homologous boosting vaccination with the ChAd3.EBOV GP vaccine resulted in decreased protection, with only 1 out of 3 NHP surviving challenge, possibly suggesting pre-existing vector immunity.[26] Nevertheless, the early success of the ChAd3.EBOV GP vaccine allowed for acceleration into human Phase I clinical trials during the 2013 West African Ebola outbreak (NCT02289027 and NCT02231866). The vaccine was well tolerated, and resulted in seroconversion, with antibody levels approaching those observed in NHP studies.[80,81] Of concern though was the durability of immunity, as humoral responses waned by 6 months post vaccination.[80] Taken collectively, the data suggest that adenovirus-vectored EBOV vaccines have promise, but continued research is needed to avoid the issues associated with host pre-existing immunity and limited duration of immunity. Additionally, the immunogenicity of adenovirus-vectored MARV vaccines remain to be tested in humans, although

a Phase I, open-label clinical study is currently underway to investigate the safety, tolerability, and immunogenicity of the ChAd3.MARV GP vaccine (NCT03475056).

Another adenovirus-based approach (CAdVax) was tested as a pan-filovirus vaccine. The CAdVax system uses multiple adenovirus constructs to express genes encoding EBOV GP, EBOV NP, SUDV GP, MARV NP, two MARV GPs (Musoke and Ci67) and RAVV GP, which can be combined into a single vaccine.[27] NHPs received two doses of the CAdVax-Panfilo vaccine and were then separated into two groups. Group 1 was challenged with a lethal dose of MARV, whereas group 2 was challenged with EBOV. All NHPs survived filovirus challenge without developing signs of disease, including systemic viremia. 10 weeks after the initial challenge, NHPs were back-challenged with heterologous filovirus strains. Group 1 was re-challenged with a lethal dose of SUDV, while group 2 was re-challenged with MARV. As with the initial challenge, all NHPs survived the re-challenge without displaying clinical symptoms of filovirus challenge. As no cross-reactive antibodies have been described for EBOV and MARV, it is believed that the CAdVax-Panfilo vaccine provided widespread protection from multiple filovirus species. In a second study, CAdVax provided protection against both EBOV and SUDV following either parenteral or aerosol challenge, even in NHPs that had previously been exposed to adenovirus vector.[28]

Vaccinia virus vectors

Vaccinia virus is a large, complex, enveloped DNA virus belonging to the *Poxviridae* family. The vaccinia vaccine was initially developed to eradicate smallpox, but in recent years it has been used as a viral vector due to its ability to accept large, foreign gene inserts. A recombinant vaccinia vector was one of the first replication competent vaccine vectors to be tested for the ability to efficiently deliver filovirus genes and elicit protective immunity against filovirus challenge. A recombinant vaccinia virus (VACV) expressing EBOV GP was tested for protective efficacy in NHPs. Three doses of the VACV-GP vaccine resulted in seroconversion and the development of neutralizing antibodies.[17] However, all NHPs succumbed to EBOV disease within 1 week of challenge, preventing this vaccine from being considered a viable option. The current iteration of VACV filovirus vaccines are based on the replication incompetent modified vaccinia Ankara (MVA) vector, and have yielded considerably more success. Two versions of the MVA-vectored vaccines have been tested: the monovalent MVA-EBOV encoding GP and the MVA-BN-Filo vaccine, a quadrivalent vaccine encoding the GP of EBOV, SUDV, and MARV, as well as the NP from Tai Forest virus, which was the first pox-vectored filovirus vaccine to be tested in human clinical trials.[82] Domi et al. showed that MVA vectors expressing Makona GP and VP40 formed self-assembling virus like particles (VLPs) that protected 100% of guinea pigs from EBOV challenge.[29] Furthermore, the rhesus macaques receiving either a prime or prime/boost of MVA-$EBOV_{Makona}$ GP/VP40 vaccine were completely protected from EBOV challenge.[29] The prime/boost regimen improved antibody titers and neutralizing antibody generation compared to the single dose, but did not significantly reduce viremia. Two Phase I clinical trials (NCT02451891 and NCT02485912) demonstrated that one dose of the MVA-EBOV vaccine alone was immunogenic, yielding anti-EBOV GP antibodies and antigen-specific IFN-γ^+ T cells.[83] When given at either 1×10^8 or 1.5×10^8 plaque forming units (PFU), vaccinees reported only mild adverse effects, most commonly headache and fatigue. Notably, vaccinees did not receive a second dose of MVA-EBOV, preventing a clear understanding of the effectiveness of a booster vaccination.

The data from MVA-EBOV clinical trials suggest that MVA-vectored filovirus vaccines can generate protective immunity in human patients, but perhaps the most effective use of MVA-vectored vaccines is as a heterologous boost to the adenovirus-vectored vaccines. Stanely et al. explored the potency of the heterologous ChAd3.EBOV prime/MVA-EBOV boost vaccination regimen in cynomolgus macaques.[26] The ChAd3.EBOV vaccine alone provided only short-term, partial protection from EBOV challenge. Boosting with MVA-EBOV further improved the anti-EBOV immune responses, increasing anti-GP antibody titers and polyfunctional T cell population proportions. The addition of a MVA-EBOV boost also extended the durability of the immune response, increasing the frequency of effector memory T cell populations. The utility of the MVA-EBOV boost became further evident as all animals were protected from viral challenge (versus 50% in the original ChAd3.EBOV study).[26] The NHP data translated to the clinic as well (NCT02451891 and NCT02485912). MVA-EBOV given as a boost to ChAd3.EBOV elicited humoral and cellular mediated immune responses that were significantly improved compared to MVA-EBOV alone.[83] Moreover, a shortened interval of only 1 week between the prime and boosting vaccinations elicited immune responses that were comparable to the more standard 4 week interval.[83] Similar results were seen in a Phase I clinical trial where MVA-BN-Filo was delivered as a boosting vaccination following ChAd3.EBOV prime (NCT02240875). In this trial, participants received either the ChAd3.EBOV vaccine alone, or in conjunction with a boosting MVA-BN-Filo vaccination between 3 to 10 weeks later.[82] Boosting with MVA-BN-Filo significantly improved the humoral response, with antibody titers increasing by day 7 post MVA-BN-Filo dosing. The response peaked at day 14 after boosting and decreased slightly by day 28. Similarly, cellular immunity improved by 7 days post MVA-BN-Filo boost, with the generation of polyfunctional $CD4^+$ and $CD8^+$ T cells peaking at this time point. Additional testing showed that a limited interval of 1–2 weeks between boosting vaccinations elicited immune responses that were comparable to the traditional 8 week interval.[82]

In addition to the success of the ChAd3.EBOV/MVA-BN-Filo studies, it has been reported that a vaccination regimen consisting of a trivalent Ad26 vaccine expressing the GPs from EBOV, SUDV, and MARV followed by a boosting MVA-BN-Filo dose improved cellular immunity and protected NHPs from EBOV challenge.[25] A subsequent Phase I clinical trial provided more evidence that an adenovirus/MVA-EBOV heterologous prime/boost approach is highly immunogenic (NCT02891980). Patients receiving an Ad26.

EBOV prime/MVA-BN-Filo boost exhibited sustained anti-EBOV antibody responses for at least 1 year post vaccination.[84,85] Moreover, 86% of vaccine recipients had Ebola-specific T cell responses. These data are in agreement with Phase I clinical studies conducted in Africa showing that Ad26.EBOV prime/MVA-BN-Filo boost can elicit humoral responses for at least a year following vaccination.[86,87] The strong immunogenicity results observed in these Phase I trials have provided a basis for further vaccine assessment in Phase II (NCT02876328 and NCT02509494) and III (NCT02543567 and NCT02543268) studies. The combined data generated in these studies suggest MVA vectored filovirus vaccines may be useful in outbreak settings when quick response ring vaccination strategies are preferable.

DNA vaccine

DNA vaccines have proven to be one of the most versatile and promising vaccine platforms. This is frequently attributed to the ability of DNA vaccines to stimulate potent innate and adaptive immune responses.[88-91] Moreover, the endogenous antigen production by DNA vaccine transfected host cells elicits potent humoral and cell-mediated immune responses. However, simple needle and syringe delivery, whether by intramuscular (IM) or intradermal (ID) injection, has displayed poor immunogenicity in NHPs and humans due to inefficient plasmid uptake by host cells. Alternative delivery methods such as electroporation (EP) or ballistic gene gun have significantly improved DNA vaccine immunogenicity by increasing the transport efficiency of plasmids across cellular membranes.[92,93] These advances suggest that the DNA vaccine platform may be an effective means of eliciting protective immunity against filovirus infection.

Xu et al. published the first DNA vaccine for EBOV.[94] This vaccine expressed EBOV GP and NP, and four doses administered by IM injection completely protected guinea pigs from EBOV challenge. Protection correlated to the generation of anti-EBOV antibodies and T cell responses directed against GP. Another study conducted in mice demonstrated 100% protection after four doses of an EBOV GP DNA vaccine delivered by gene gun.[95] Suschak et al. recently reported that only two doses of EBOV GP delivered by IM injection is required for complete protection of mice from EBOV challenge, and that immunogenicity can be improved with the addition of genetic adjuvants to the vaccine formulation.[96] Likewise, priming with a DNA vaccine expressing EBOV GP and boosting with a soluble GP (sGP) isoform expressing DNA vaccine elicited antibodies against GP and sGP, while providing complete protection in mice without signs of illness.[97] Subsequent work has shown the protective efficacy of pan-filovirus DNA vaccines. Shedlock et al. demonstrated that a trivalent vaccine composed of plasmids expressing consensus sequences from EBOV GP, SUDV GP, and MARV GP protects both mice and guinea pigs from EBOV and MARV challenge when delivered by IM-EP.[98] Likewise, Grant-Klein et al. demonstrated that IM-EP administration of a quadrivalent filovirus DNA vaccine expressing the GPs of EBOV, SUDV, MARV, and RAVV was protective against both EBOV and RAVV challenge in mice without evidence of immune interference.[99]

Complete protection against filovirus challenge has been achieved in mice and guinea pigs, but NHP studies have proven less successful. Riemenschneider et al. demonstrated 67% protection against MARV challenge in two independent NHP studies following gene gun vaccination with a DNA vaccine expressing MARV GP.[47] In these studies, all animals, including survivors, developed clinical signs of disease within 10 days of challenge, suggesting room for improvement. To address this, researchers have sought to improve DNA vaccine design. One such method is codon optimization of gene inserts to match the codon usage of the available tRNA pool within the host species. Codon optimization has been shown to improve mRNA half-life, increase translation efficiency, and enhance gene expression.[100] IM-EP vaccination with codon-optimized, monovalent filovirus DNA vaccines protected 5/6 NHPs from homologous EBOV or MARV challenge.[30] A synthetic plasmid vaccine expressing a GP consensus sequence from multiple West African $EBOV_{Makona}$ isolates had similar efficacy in NHPs following IM-EP.[31] However, the quadrivalent filovirus vaccine described by Grant-Klein et al. could only protect against MARV, with 1/5 NHPs surviving EBOV challenge.[30] The reason(s) for this failure in protection have yet to be elucidated.

EP and gene gun are highly immunogenic, but can be cumbersome to deploy in areas where EBOV and MARV are endemic. This has led researchers to investigate other approaches for improving DNA vaccine efficacy in humans without the use of specialized delivery devices. Needle-free jet-injection systems have proven suitable for DNA vaccine delivery, particularly when paired with a heterologous prime/boost vaccination strategy. One of the first EBOV vaccination strategies to prove efficacious in NHPs was a DNA vaccine provided in conjunction with a recombinant Ad5.EBOV GP vaccine.[42] Hensley and colleagues showed that a DNA prime/Ad5 vaccine expressing EBOV GP and SUDV GP elicited cross-protective immunity in cynomolgus macaques.[43] A study published the same year showed improved efficacy against MARV challenge in NHPs. Heterologous vaccination with DNA plasmids and Ad5 vectors expressing $MARV_{Angola}$ GP generated anti-MARV GP IgG and T cell responses.[46] The high levels of GP-specific immunity limited the development of clinical disease, with only mild rash, lymphopenia, and anorexia recorded after challenge. Additionally, none of the NHPs developed detectable levels of viremia.

Multiple clinical trials have been conducted to evaluate the safety and immunogenicity of filovirus DNA vaccines. Martin et al. demonstrated that a three plasmid DNA vaccine expressing EBOV GP, EBOV NP, and SUDV GP was well tolerated in human patients (NCT00072605).[101] Antigen-specific antibodies to at least one of the expressed antigens were detected in all vaccinees. Additionally, 20/20 vaccinees developed anti-GP $CD4^+$ T cells, and 6/20 vaccinees had $CD8^+$ T cell responses directed against GP and NP, providing further evidence that DNA vaccination can elicit both humoral and cellular immunity in a clinically relevant filovirus vaccine. Another Phase I clinical trial (NCT00605514) initiated in 2008 established the immunogenicity of two separate DNA

vaccines, one encoding $MARV_{Angola}$ GP and the second encoding EBOV and SUDV GP.[102] This study showed that a 3 dose vaccination regimen of either vaccine was immunogenic and well tolerated, with patients developing humoral and cellular immune responses. A fourth homologous dose further boosted antibody titers and T cell responses. Finally, a Phase I, double-blinded, randomized, placebo-controlled clinical trial was conducted in 2009 in Kampala, Uganda (NCT00997607). This was the first vaccine clinical trial to be conducted in Africa for Ebola and Marburg.[103] 108 participants were enrolled in this two part study. In part one, participants were randomly assigned to one of three groups: EBOV GP only, MARV GP only, or placebo. In part two, participants were randomly assigned to receive both vaccines, EBOV GP in the left arm and MARV GP in the right, or placebo vaccination in both arms. The results showed that when given separately or as a combination, both vaccines were well tolerated, safe, and immunogenic. Vaccinees receiving one or both of the filovirus vaccines developed antigen-specific humoral and cellular immune responses, suggesting limited immune interference.

VLP

Filovirus virus like particles (VLPs) are safe, non-replicating vaccines generated by co-expression of the GP and structural matrix protein VP40 in mammalian cells or insect cells.[48,104-106] VLPs self-assemble within the cell and bud from the host cell surface. EBOV VLPs are morphologically similar to EBOV particles.[32,107] VLPs containing filovirus GP have successfully been used to vaccinate rodents, even in the absence of adjuvants.[108] The addition of adjuvants to filovirus VLP formulations have improved immunogenicity and allowed for reduction in vaccine dose. VLPs delivered in combination with QS-21[109] or RIBI adjuvant[53,110] completely protect mice and guinea pigs against challenge with either EBOV or MARV, even after a single dose. Other filovirus proteins such as VP24 or NP may be incorporated into the VLP, providing broader immune responses. NP is frequently added to EBOV VLP vaccines, as multiple studies have shown that anti-EBOV NP antibodies can protect mice from lethal EBOV challenge.[95,111]

The protective efficacy of VLPs seen in rodent models has generally also been observed in NHPs. Warfield and colleagues achieved complete protection of NHPs from lethal intramuscular EBOV challenge.[32] NHPs were vaccinated three times with 250 μg of EBOV VLPs composed of EBOV GP, VP40, and NP. Inclusion of 0.5 ml of RIBI adjuvant allowed for a significant reduction in the dose of EBOV VLPs required for protection.[110] Immunized guinea pigs never developed clinical signs of infection, and no viremia could be detected at any time point following challenge. In a follow-up NHP study, Warfield et al. compared the protective efficacy of a "triple" VLP composed of EBOV GP, VP40, and NP to a "double" VLP consisting of EBOV GP and VP40. In this study, inclusion of NP decreased anti-EBOV GP IgG levels, but provided complete protection from viral challenge.[33] Likewise, MARV VLPs expressing only GP or GP and NP protected against three strains of MARV; MARV, $MARV_{Ci67}$, and RAVV.[48] Recently, Dye et al. demonstrated that MARV VLPs adjuvanted with QS-21 or poly I:C can protect NHPs against MARV aerosol challenge.[49]

While several NHP studies have yielded encouraging protective efficacy results for homologous EBOV or MARV challenge, attempts to produce a pan-filovirus VLP vaccine have been inconsistent. VLPs expressing a trimeric hybrid of EBOV, SUDV, and MARV GP provided protection from MARV challenge, but only partial protection against EBOV challenge in guinea pigs.[112] These findings are in agreement with reports that EBOV and SUDV offer little in terms of cross-protective immunity.[20]

The multitude of advantages afforded by VLPs make them a promising vaccine platform. The ease of production and ability of VLPs to avoid host pre-existing immunity has led to commercial development. For instance, Novavax, Inc. is currently testing an $EBOV_{Makona}$ GP VLP in a Phase I, randomized, dose-ranging, clinical trial (NCT02370589). This vaccine includes Novavax, Inc.'s proprietary adjuvant Matrix-M, which has been shown to improve EBOV protective efficacy in mice[113] and NHPs.[114] Early safety and immunogenicity results from this trial are encouraging, as all vaccinees receiving the adjuvanted vaccine seroconverted.[114] The NHP and clinical study data suggest a possible path forward for filovirus VLP vaccines.

Replication competent vaccines

Several promising filovirus vaccine candidates have been developed from replication competent viral vectors. Vectors derived from recombinant cytomegalovirus, human parainfluenza virus 3, and rabies virus have been tested. The most prominent replicating vector is the recombinant vesicular stomatitis virus, which has been investigated in multiple clinical trials. Replication competent vaccines generally have several advantages over non-replicating ones, particularly increased immunogenicity and durable immunity. However, replication competent vaccines present concerns over reversion to a pathogenic virus, pre-existing immunity to the vector, and adverse effects in immunocompromised patients.

Recombinant cytomegalovirus

Cytomegalovirus (CMV) is a widely distributed β-herpes virus that infects humans. CMV infection is persistent, but typically asymptomatic, in healthy adults. CMV has recently gained considerable interest as a vaccine vector due to its strict species-specificity and continuous replication within the host.[115-117] CMV can infect, and re-infect, a host regardless of pre-existing immunity.[118] Additionally, CMV vectors induce large T cell responses, making them ideal for eliciting cellular immunity.[119] In a proof of concept study, Tsuda et al. constructed a mouse replicating CMV vector that expressed EBOV NP (MCMV/EBOV NP_{CTL}). MCMV/EBOV NP_{CTL} induced high levels of EBOV NP-specific $CD8^+$ T cells, but failed to elicit EBOV neutralizing antibodies in mice. However, all vaccinated animals survived EBOV challenge. A follow-on study showed that a single dose of MCMV/EBOV NP_{CTL} provided durable protective immunity for a period of 119 days following vaccination.[120]

In a recent NHP study, Marzi et al. tested a recombinant rhesus macaque CMV (RhCMV) vaccine expressing codon optimized EBOV GP.[34] A prime/boost vaccination scheme elicited GP-specific antibodies and protected 3/4 (75%) of macaques from lethal EBOV challenge. The surviving NHPs exhibited mild signs of disease, but viremia kinetics were delayed and never reached the level seen in control animals. Regardless of the lack of complete protection, the ability to generate an immunogenic, disseminating vaccine suggests that CMV vectored filovirus vaccines have great potential.

Human parainfluenza virus type 3

Another promising vector based platform is human parainfluenza virus type 3 (HPIV3). HPIV3 is a negative-sense RNA virus of the *Paramyxoviridae* family that causes respiratory disease in pediatric patients. HPIV3, as an infectious agent or when used as a vector, induces systemic and localized respiratory tract immune responses.[121] Bukreyev and colleagues initially inserted a transcription cassette expressing either EBOV GP or EBOV GP and EBOV NP into HPIV3. A single intranasal administration of this vaccine protected guinea pigs from challenge with 1000 PFU EBOV.[122] A single dose delivered by combined intranasal/intratracheal administration is immunogenic in African green monkeys,[123] and provided 88% protection in a rhesus macaque model.[35] A second HPIV3/EBOV GP dose was required to achieve 100% protective efficacy.[35] Interestingly, aerosolized delivery of HPIV3/EBOV GP induces high levels of not only systemic immunity, but also mucosal immunity. Meyer et al. reported elevated levels of IgG and IgA in vaccinated rhesus macaques.[36] Furthermore, antigen-specific $CD8^+$ T cells were isolated from the lung, and may have contributed to protection from aerosol EBOV challenge. Even with the strong NHP efficacy data, the possibility of host pre-existing HPIV3 immunity has impeded the transition of the HPIV3/EBOV vaccine into humans.[124,125] To combat this, Bukreyev and colleagues generated an attenuated HPIV3 vector wherein the main targets for HPIV3-specific humoral responses, the F and HN genes, have been removed (HPIV3/ΔF-HN/EBOVGP).[126] The new attenuated vector proved to be more immunogenic than the original construct, inducing a 6.4 fold increase in anti-GP ELISA titers compared to HPIV3/EBOV GP.[126] As studies have shown that HPIV3/EBOV GP is safe and not associated with increased vector replication in the respiratory tract,[121] a Phase I clinical trial was recently conducted in the US to evaluate the safety, infectivity, and immunogenicity of the HPIV3/EBOV vaccine administered intranasaly (NCT02564575). Results for NCT02564575 have not been released yet, but a follow on Phase I has been initiated for the HPIV3/ΔF-HN/EBOVGP vaccine (NCT03462004).

Recombinant rabies virus based vaccines

Rabies virus (RABV) vectors have been explored by several groups as a vaccine platform against EBOV. RABV is a non-segmented, negative-stranded RNA virus belonging to the *Rhabdoviridae* family. EBOV vaccines produced using a RABV vector are replication competent. The vaccine is constructed by replacing the RABV glycoprotein of strain SAD B19 with EBOV GP (BNSP333-GP). This reduces RABV neurovirulence, as early testing in mice has not shown active RABV infection.[62,127] Two other RABV/EBOV GP vaccines have been generated: a replication incompetent vector and a chemically inactivated version produced by expressing EBOV GP in a reverse genetics system.[127,128] A similar RABV vectored vaccine has been generated for MARV and tested in mice.[129] RABV vectors have been used as effective vaccines for HIV, SARS-CoV, and hepatitis C virus.[37]

Studies in mice[127] and NHPs[37] have shown that the RABV/EBOV GP vaccine is safe, immunogenic, and protective. One dose of the replication competent vector fully protected NHPs from lethal EBOV challenge 70 days after vaccination.[37] Two doses of the replication incompetent and chemically inactivated RABV/EBOV GP vaccines only provided 50% protection, most likely due to the decreased level of IgG1 antibodies elicited compared to the attenuated RABV/EBOV GP. To improve protection, codon-optimized EBOV GP was inserted into the BNSP333 RABV vector (BNSP333-coEBOV GP). Two doses of this vaccine were sufficient to protect all NHPs from challenge with 100 PFU EBOV.[38] The BNSP333-coEBOV GP vaccine was recently tested for the possibility of oral delivery. Vaccinated chimpanzees developed EBOV-specific immune responses, to include neutralizing antibodies, highlighting the potential for use as a wildlife vaccine.[130] The success of pre-clinical studies has led the National Institutes of Allergy and Infectious Disease (NIAID) to pursue the development of replication competent RABV/filovirus vaccines (NIAID Contract No. HHSN272201700082C).

VSV

The final filovirus candidate is also one of the most encouraging. Recombinant vesicular stomatitis virus (rVSV) is a negative-strand, RNA virus of the *Rhabdoviridae* family. Its genome encodes five proteins: G protein, large protein, phosphoprotein, matrix protein, and nucleoprotein. The VSV G protein is expressed on the surface of the virion, and enables viral entry.[131] A reverse genetics system developed by Rose and colleagues allows for expression of foreign antigenic proteins from the VSV vector.[132,133] The ease with which VSV can be grown to extremely high titers *in vitro* and *in vivo* make it an optimal vaccine vector. The VSV vector has also been shown to be a potent inducer of innate and adaptive immune responses.[134] These advantages prompted Garbutt et al. to produce the first rVSV expressing $EBOV_{Mayinga}$ GP, $MARV_{Musoke}$ GP, or $Lassa_{Josiah}$ virus GP.[135] For the rest of this review, we will refer to these vaccines as VSV-EBOV or VSV-MARV. The rVSV vector has been tested in numerous immunogenicity and protective efficacy studies. Here we will present only a brief overview of the data with a particular focus on pre-clinical NHP studies and clinical trials. Further information is reviewed elsewhere.[62,136,137]

The first NHP efficacy study conducted with the rVSV was performed by Jones et al in 2005. In this study, a single dose of VSV-EBOV or VSV-MARV protected all NHPs challenged

28 days after vaccination.[39] Notably, no rVSV shedding was detected in vaccinated NHPs, and none of the animals developed clinical signs of disease. Interestingly, NHPs vaccinated with VSV-EBOV developed both humoral and cellular immune responses, but the VSV-MARV induced primarily humoral immunity. After the initial challenge, all survivors were back-challenged with heterologous virus strains. All animals initially challenged with MARV were rechallenged with the related $MARV_{Popp}$ strain. Complete protection was observed. Conversely, 75% of the EBOV survivors succumbed to SUDV rechallenge. To address the lack of cross-protection, Geisbert et al blended VSV-MARV, VSV-EBOV, and VSV-$SUDV_{Boniface}$ into a trivalent single dose vaccine.[40] Falzarano et al. subsequently demonstrated that a single vaccination with VSV-EBOV provided 75% cross-protection against BDBV challenge.[41] These studies demonstrated for the first time that cross-protective filovirus immunity is achievable with a single VSV-vectored vaccine.

A number of pre-clinical studies have established that rVSV filovirus vaccines can rapidly induce protective immune responses in NHPs.[40,138] To investigate the potential use of the VSV-EBOV vaccine as a post-exposure emergency treatment, Feldmann et al. vaccinated guinea pigs and mice 24 hours after EBOV challenge.[139] This resulted in 50% protection in guinea pigs and 100% protection in mice. More importantly, administration of VSV-EBOV (50%) or VSV-SUDV (100%) can protect cynomolgus macaques up to 30 minutes after challenge.[139,140] Similar protection levels were seen in rhesus macaques vaccinated either 24 (83%) or 48 (33%) hours post MARV challenge.[141]

The 2013 West African EBOV outbreak prompted several Phase I-III clinical trials in an attempt to develop effective countermeasures. The first two Phase I trials were dose-escalation studies conducted in the USA and designed to test the safety and immunogenicity of either a single dose or two identical doses of VSV-EBOV (NCT02269423 and NCT02280408).[142] Vaccinees received either 3×10^6, 2×10^7, or 1×10^8 PFU of the vaccine or placebo. The VSV-EBOV vaccine did not appear to cause serious adverse events. Some vaccinees exhibited mild to moderate adverse events such as headache, fatigue, myalgia, arthralgia, fever, and chills; symptoms that are consistent with other replicating vaccines. Most of these symptoms resolved by day 4 post vaccination. The second dose appeared to be less reactogenic than the first. Importantly, all vaccinees seroconverted by day 28, with those patients in the 2×10^7 and 1×10^8 PFU dose groups generating higher levels of EBOV GP-specific antibodies as measured by ELISA and neutralization assay. Increasing the dose from 2×10^7 to 1×10^8 PFU did not provide a significant improvement in vaccine immunogenicity. Administration of a second VSV-EBOV dose provided only a transient increase in geometric mean antibody titers, as antibody responses waned by two months post second vaccination. Notably, these trials identified novel EBOV GP epitopes and showed that IgM contributes substantially to virus neutralization .[143]

Three open-label dose-escalation Phase I trials were conducted in Europe (Hamburg, Germany; Geneva, Switzerland) and Africa (Lambaréné, Gabon; Kilifi, Kenya) almost simultaneously with the American studies (NCT02283099, NCT02287480, NCT02296983). Vaccinees were administered doses ranging from 3×10^5 to 5×10^7 PFU VSV-EBOV or a saline placebo. As with the American studies, the preliminary results from these three studies demonstrated good immunogenicity, with vaccinees developing neutralizing antibodies following one vaccination.[144,145] Mild to moderate reactogenicity was frequent, but generally transient; however, 11 of 51 participants in Geneva developed arthritis 2 weeks post vaccination. 8 of these participants received a dose of 1×10^7 PFU while the remaining 3 participants received 5×10^7 PFU. These data suggest that a lower vaccine dose may be necessary to avoid systemic adverse events. The reactogenicity issues necessitated a resumption of the Geneva trial with the addition of a new participant cohort at a dose of 3×10^5 PFU.[146] Although this was a setback, it allowed for comparison of safety and immunogenicity at various doses. The reduction in dose decreased the occurrence of arthritis, but 25% of the new participants still reported arthralgia following vaccination. Moreover, the reduction in dose negatively impacted vaccine immunogenicity. Despite similar seroconversion rates, vaccinees receiving the lower VSV-EBOV dose had significantly reduced levels of EBOV GP-specific IgG and neutralizing titers, suggesting that dose reduction strategies may be suboptimal.[146] Of particular interest, longitudinal analysis showed that one-third of vaccinees developed anti-VSV adaptive immune responses. 28% of vaccines developed VSV-M-specific antibodies, with the 3×10^5 group having the highest magnitude of anti-VSV-M IgG titers. In addition, 36% of vaccinees developed VSV-N-specific cellular immunity.[147] Although the lack of data on VSV-directed immunity in humans precludes definitive analysis, these results highlight the potential for problems with homologous VSV vector boosting.

Some of the most useful data obtained from the European and African trials centered on how VSV-EBOV vaccination shapes the immune response. Rechtien et al. used a systems vaccinology approach to investigate the innate immune response triggered by VSV-EBOV. These studies showed that vaccination-induced natural killer (NK) cell activation contributes to control of viral replication, and that NK cell frequency correlated with antibody responses.[148] Huttner et al. reported that increases in CCL2, CCL4, IL-6, TNF-α, IL-1Rα, and IL-10 correlated with development of arthritis in vaccinees.[145] Finally, in depth sample analysis showed that antibody responses correlated to the induction of circulating T follicular helper (T_{fh17}) cells.[149]

In 2015, three VSV-EBOV Phase III clinical trials were initiated. The first clinical trial conducted in Guinea was an open-label, cluster-randomized ring vaccination study.[150,151] In this trial, contacts and contacts of contacts of EBOV positive patients were divided into two groups and vaccinated with a single 2×10^7 dose of VSV-EBOV either immediately (4123 patients) or 21 days later (3528 patients). No patients receiving immediate vaccination developed EBOV disease, proving that the VSV-EBOV vaccine can quickly elicit anti-EBOV protective immunity. Conversely, 16 cases were reported in patients vaccinated after the 21 day delay, although all patients survived development of disease. This

study provided critical evidence that a ring vaccination strategy may be effective during EBOV outbreaks, but that optimal protection requires immediate vaccine administration.

The second trial was an open-label, individually randomized controlled Phase II/III trial conducted in Sierra Leone completed in December 2016 (NCT02378753). Out of 8,000 enrolled participants, 64 developed illness that was suspected of being EBOV disease but no laboratory confirmed cases were identified. Vaccination did not result in any reported serious adverse events, including arthritis or hospitalization.[152] The third Phase III VSV-EBOV study initiated in 2015 was a randomized, double-blind, multicenter trial conducted in the USA, Spain, and Canada with 1197 total participants (NCT02503202). The goal of this study was to assess the safety and immunogenicity of three consistency lots (2×10^7 PFU) and a high-dose lot (1×10^8). The vaccine was well-tolerated with only minor adverse events reported, and no vaccine-related severe adverse events reported.[153]

A recent Phase II randomized, double-blind, placebo controlled, three-arm clinical study was conducted in Liberia to compare the immunogenicity of both the ChAd3.EBOV vaccine and VSV-EBOV to a placebo (NCT02344407). Both vaccines proved to be immunogenic in this study, with 63.5% of ChAd3.EBOV vaccinees and 79.5% of VSV-EBOV vaccinees having measurable antibody titers 12 months post vaccination.[154] Both vaccines appeared to be well-tolerated, with most vaccine-related symptoms resolving within the first month. In addition, a small subgroup of participants was positive for HIV. None of the HIV^+ participants reported a serious adverse event within 1 month of the first vaccination, and there was no statistically significant increase in adverse events over the course of the 12 month study compared to the placebo group.[154] However, a lower proportion of HIV^+ individuals generated anti-EBOV antibodies than did HIV^- participants. The results of NCT02344407 may be confirmed in a Phase II study that is currently ongoing. NCT03031912 is a randomized, placebo-controlled, multisite, double-blind trial that will test the safety of VSV-EBOV in HIV infected adults. Participants will be recruited from two Canadian sites and two African countries, Burkina Faso and Senegal. Should the VSV-EBOV vaccine prove safe in these studies, it will lend support to the use of VSV-EBOV as an emergency countermeasure for use during outbreaks.

Recently, an open-label, dose-escalation trial to assess the safety, side-effects, and immunogenicity of a heterologous prime/boost regimen consisting of the VSV-EBOV and Ad5.EBOV GP vaccines (GamEvac-Combi) was conducted in 84 healthy adults living in the Russian Federation.[155] Patients received either a half dose of the VSV-EBOV (12.5×10^6 PFU) or Ad5.EBOV GP (12.5×10^{10} PFU), a half dose of the VSV-EBOV followed by a half dose boost of Ad5.EBOV GP 21 days later, or a full dose prime/boost of VSV-EBOV /Ad5.EBOV GP (2.5×10^7 PFU and 2.5×10^{11} PFU respectively). Following vaccination, 100% of vaccinees seroconverted, with those receiving the Ad5.EBOV GP boost having broadly improved immune responses. Patients receiving the heterologous prime/boost exhibited increased anti-EBOV GP titers compared to patients receiving VSV-EBOV alone. 93% of vaccinees receiving the full dose heterologous prime/boost developed anti-EBOV neutralizing antibodies. Vaccinees also developed anti-EBOV GP T cells responses, with $CD4^+$ and $CD8^+$ T cell populations peaking by day 28, and decreasing by day 42. Cellular immunity results were similar to those seen in previous ChAd3.EBOV clinical trials.[156] Two follow on clinical trials (NCT02911415 and NCT03072030) are currently underway to test the immunogenicity and duration of immune responses of the VSV-EBOV/Ad5.EBOV GP regimen.

Conclusion

The 2013 West African EBOV outbreak was a watershed moment in the development of filovirus vaccines. In the forty years prior, very few prophylactic or therapeutic countermeasures were developed for EBOV and MARV. The lack of research was partly due to limited funding mechanisms and a lack of commercial interest. However, the 2013 EBOV epidemic changed the paradigm, highlighting the need for safe, effective vaccines that can be speedily deployed. Clinical trials were rapidly accelerated for lead vaccine candidates, and funding became available for the development of next-generation prophylactics. We now have a significant amount of Phase I and Phase II data from a number of vaccines in diverse populations. The monitoring of safety and efficacy data allowed for the initiation of the first EBOV Phase III vaccine trial. The Phase III VSV-EBOV trials established the protective efficacy of a ring vaccination strategy in an outbreak setting. In spite of the unpreparedness of the healthcare and scientific community, important lessons were learned that may help prevent future outbreaks from reaching the same magnitude.

Still, huge challenges remain. Licensing of a vaccine that can quickly be manufactured and deployed in an outbreak setting remains an unmet goal. We still do not have a clear understanding of the immunological correlates of filovirus vaccine protection. The most advanced vaccine, VSV-EBOV, demonstrated efficacy in Phase III trials, but concerns linger as to the utility of VSV-EBOV in a large scale vaccination campaign. More data is needed to confirm the long-term durability of immunity elicited by VSV-EBOV, as several studies have shown that anti-EBOV responses may fade within a few months. Additionally, the immunogenicity of VSV-EBOV in immunocompromised vaccinees remains unclear, as HIV^+ vaccinees exhibited impaired antibody responses. Moreover, the logistical issues that previously hindered filovirus vaccine development endure. While a single shot vaccine may partially address logistical concerns, the immunogenicity demonstrated by prime/boost studies suggest that this may be a more realistic approach. Despite these challenges, we have come a long way and are better prepared now then we were in 2013.

Acknowledgments

John J. Suschak and Connie S. Schmaljohn would like to acknowledge funding from the Joint Science and Technology Office for Chemical and Biological Defense of the Defense Threat and Reduction Agency. The

opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily indorsed by the U.S. Army.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Defense Threat and Reduction Agency [CB10204].

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